THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 16, Issue of April 22, pp. 16157–16162, 2005

Printed in U.S.A.

Metal Ion-dependent Effects of Clioquinol on the Fibril Growth of an Amyloid β Peptide*

Received for publication, January 10, 2005, and in revised form, February 16, 2005
Published, JBC Papers in Press, February 16, 2005, DOI 10.1074/jbc.M500309200

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Although metal ions such as Cu^{2+}, Zn^{2+}, and Fe^{3+} are implicated to play a key role in Alzheimer disease, their role is rather complex, and comprehensive understanding is not yet obtained. We show that Cu^{2+} and Zn^{2+} but not Fe^{3+} renders the amyloid β peptide, $A\beta_{1-40}$, non-fibrillogenic in nature. However, preformed fibrils of $A\beta_{1-40}$ were stable when treated with these metal ions. Consequently, fibril growth of $A\beta_{1-40}$ could be switched on/off by switching the molecule between its apo- and holo-forms. Clioquinol, a potential drug for Alzheimer disease, induced resumption of the Cu^{2+}-suppressed but not the Zn^{2+}-suppressed fibril growth of $A\beta_{1-40}$. The observed synergistic effect of clioquinol and Zn^{2+} suggests that Zn^{2+}-clioquinol complex effectively retards fibril growth. Thus, clioquinol has dual effects; although it disaggregates the metal ion-induced aggregates of $A\beta_{1-40}$ through metal chelation, it further retards the fibril growth along with Zn^{2+}. These results indicate the mechanism of metal ions in suppressing Aβ amyloid formation, as well as providing information toward the use of metal ion chelators, particularly clioquinol, as potential drugs for Alzheimer disease.

Alzheimer disease (AD)[1] is a neurodegenerative disease characterized by cerebral deposits of extracellular amyloid plaques, intracellular tangles, and intravascular or extravascular deposits (1, 2). The deposits are comprised of a mixture of 39–43-amino-acid polypeptides generally designated as amyloid-β (Aβ) peptide (3, 4). Aβ peptides are secreted by proteolytic processing of several membrane proteins, which are alternative splicing products of the gene coding the amyloid precursor protein (APP) (5–7). Accumulation of the Aβ peptides as insoluble amyloid deposits occurs in AD due to yet unclear mechanism(s).

The role of metal ions, notably Cu^{2+}, Zn^{2+}, and Fe^{3+}, in AD and metal ion chelators as therapeutic agents has been the subject of interest over recent years (8–10). Supplementing Cu^{2+} in drinking water leads to development of the disease in rabbit model (11). High concentrations of Cu^{2+} (0.4 mM) and Zn^{2+} (1 mM) have been reported in the senile plaques of the diseased brain, extracellular Cu^{2+} concentration reaching to 15 μM (12). In contrast, Cu^{2+} has been reported to decrease Aβ deposits in APP23 transgenic mice (13). Moreover, overexpression of human Aβ peptides in transgenic mice leads to a decrease in brain Cu^{2+} (14). APP knock-out mice show an increase in Cu^{2+} (15). Thus, the role of metal ions is rather complicated; it is not clear whether the accumulation of metal ions is the cause or the consequence of AD or a biological defense response to decrease progression of the disease.

The Aβ peptides bind Cu^{2+} and Zn^{2+} with high affinity (16–21). Although histidine residues, especially His-13, are involved in binding Cu^{2+} and Zn^{2+} (20–22), Cu^{2+} and Zn^{2+} differ in their coordination to His-13 (22). Cu^{2+} and Zn^{2+} trigger aggregation and precipitation of Aβ peptides (19, 23–28), which are distinct from the amyloid fibrils, especially in the β-sheet content (27, 28). Toxicity of granular aggregates is a target of recent extensive studies (29, 30). In this respect, the use of "metal-protein attenuation compounds" is proposed as a potential strategy for treating AD (31, 32). The antibiotic, clioquinol (CQ), which is also known to be a transition metal ion chelator (33, 34), has been found to dissolve deposits of the AD brain tissue (8) and to yield promising results in an animal model for AD in decreasing the number of deposits (8, 36). CQ has been the focus of attention as a potential drug (8). However, its effect on the amyloid fibril formation, especially in conjunction with Cu^{2+} or Zn^{2+}, has not been understood so far. Therefore, it is important to obtain comprehensive understanding of the interactions with metal ions and their impact on the aggregation and amyloid fibril formation of Aβ peptides.

In the present study, we investigated the role of some metal ions on the aggregation and fibrillogenic propensities of $A\beta_{1-40}$. The results show that Cu^{2+} and Zn^{2+}, but not Fe^{3+}, render $A\beta_{1-40}$ less fibrillogenic or non-fibrillogenic under conditions in which no significant metal ion-induced aggregation occurs. We used this system to examine the effects of metal ion chelators, EDTA and CQ, on the fibril growth of $A\beta_{1-40}$. The results revealed the dual effects of CQ, explaining the underlying mechanism of clioquinol as a potential drug for Alzheimer disease.

EXPERIMENTAL PROCEDURES

Materials—Human $A\beta_{1-40}$ was purchased from the Peptide Institute, Inc. (Osaka, Japan). $CuCl_2$ dihydrate, anhydrous $ZnCl_2$, and anhydrous $FeCl_3$ were purchased in their purest forms from Aldrich. CQ (5-chloro-7-iodo-8-hydroxyquinoline, commonly known as clioquinol)

* This work was supported in part by Grants-in-aid for Priority Areas (15076206, 15032228) and scientific Research (B) (13480219) from the Japanese Ministry of Education, Culture, Sports, Science and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by a Post Doctoral Fellowship from the Japan Society for Promotion of Science.

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[1] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β; APP, amyloid precursor protein; CQ, clioquinol; ThT, thioflavin T; TIRFM, total internal reflection fluorescence microscopy.

was procured from Calbiochem. "Protein assay Coomassie Brilliant Blue solution" was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Effects of CQ and Buffer Conditions on Metal Ion-induced Aggregation of Aβ$_{1-40}$—50 μM Aβ$_{1-40}$ in the indicated buffers in the presence or absence of indicated substances was incubated at 37 °C for 15 min. The final concentrations of Cu^{2+} or Zn^{2+} in the samples were 50 μM. Stock CQ solution was prepared in methanol, and it was added to a final concentration of 100 μM. The samples were centrifuged at 13,000 × *g* for 15 min. Peptide concentration in the supernatant was determined by the Bradford method using the commercial protein assay Coomassie Brilliant Blue solution and was represented as a percentage of recovery relative to the control without metal ion and CQ. We did not measure the trace contamination of the metal ions in our buffer system or from the peptide preparations. Therefore, the mentioned concentrations of the metal ions represent the added concentrations of the metal ions. Unless otherwise mentioned, the final concentration of methanol in our experiment due to the addition of CQ did not exceed 2%.

Atomic Force Microscopy—Samples of 50 μM Aβ$_{1-40}$ in 20 mM HEPES buffer, pH 7.4, containing 100 mM NaCl and 50 μM metal ions were incubated for 5 min. An aliquot of the sample was placed on a mica surface and incubated for 1 min. Excess sample was drained and washed quickly with water to remove salts. The mica surface was air-dried. Atomic force microscopy imaging was performed using a dynamic force microscope, SPI3700-SPA300, Seiko Instruments. The scanning tip was a silicon microcantilever (SI-DF20, Seiko Instruments, spring constant = 13 newton/m, resonance frequency = 135 kHz). The scan rate was 0.5 Hz.

Amyloid Fibril Growth of Aβ$_{1-40}$—Samples of 50 μM (~0.22 mg/ml) Aβ$_{1-40}$ in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl (referred to as buffer A henceforth) in the absence and presence of required concentrations of metal ions were incubated at 37 °C with 5 μg/ml sonicated fibrils (fibril seed). Aliquots (5 μl) of the samples were withdrawn at different time points and added to 1 ml of 5 μM thioflavin T in 50 mM glycine-NaOH buffer, pH 8.5 (ThT solution). Fluorescence intensity at 485 nm, which is proportional to the extent of fibril-bound ThT (37) and thus the amyloid fibril growth, was measured with an excitation wavelength of 445 nm using a Hitachi F-4500 fluorescence spectrophotometer. To test the effect of EDTA and CQ, the required concentrations of the chelators were added at the indicated time points.

Total Internal Reflection Fluorescence Microscopy (TIRFM)—ThT was added to the samples in buffer A to a final concentration of 5 μM, and the sample was placed on a glass slide. The TIRFM system to observe ThT-bound amyloid fibrils was developed based on an inverted microscope (IX70; Olympus, Tokyo, Japan) as described earlier (38, 39).

Circular Dichroism—Samples of 0.22 mg/ml Aβ$_{1-40}$ peptide alone in buffer A and the samples of the peptide and fibril seeds (5 μg/ml) incubated in the absence or presence of required concentrations of metal ions at 37 °C for 80 min were prepared. These samples were diluted 1:1 (v/v) with buffer A, and the far UV CD spectra were recorded using a Jasco-600 spectropolarimeter at 37 °C with thermo-stated cell holder and a 0.1-cm path length quartz cuvette. The data shown are the mean residue ellipticity.

Sedimentation Velocity Measurements—Sedimentation velocity measurements were performed using an Optima XL-I analytical ultracentrifuge (Beckman Coulter) with An-60 rotor and two-channel charcoal-filled Epon cells. Samples (0.3 ml) of 30 μM Aβ$_{1-40}$ in buffer A in the absence and in the presence of 50 μM metal ions or 100 μM CQ were incubated at 37 °C for 15 min followed by centrifugation at the same temperature at 224,000 × *g*. The protein boundary was scanned at 12-min intervals for its absorbance at 225 nm. The boundary curves at every 24-min intervals are shown. The sedimentation data were analyzed using the software Origin 4.1 (OriginLab).

RESULTS

Effects of CQ and Buffer Conditions on Metal Ion-induced Aggregation of Aβ$_{1-40}$—In HEPES buffer containing 100 mM NaCl (pH 6.6 or 7.4), both 50 μM Zn^{2+} and 50 μM Cu^{2+} largely decreased the percentage of recovery of Aβ$_{1-40}$ (Fig. 1, *A* and *B*). The Cu^{2+}-induced decrease in the percentage of recovery was more significant at pH 6.6 than at pH 7.4, indicating the pH dependence of the Cu^{2+} effects. Although 100 μM CQ did not itself exhibit notable effect on the aggregation of Aβ$_{1-40}$, it suppressed the metal ion-induced aggregation of the peptide, increasing its recovery (Fig. 1, *A* and *B*). Thus, in HEPES buffer, Cu^{2+} and Zn^{2+} induce aggregation of Aβ$_{1-40}$, in agreement with the earlier studies (19, 23–28), which can be suppressed by CQ. The atomic force microscopy images (Fig. 1*D*) indicated that metal ion-induced aggregates are amorphous in nature, supporting earlier reports (27, 28).

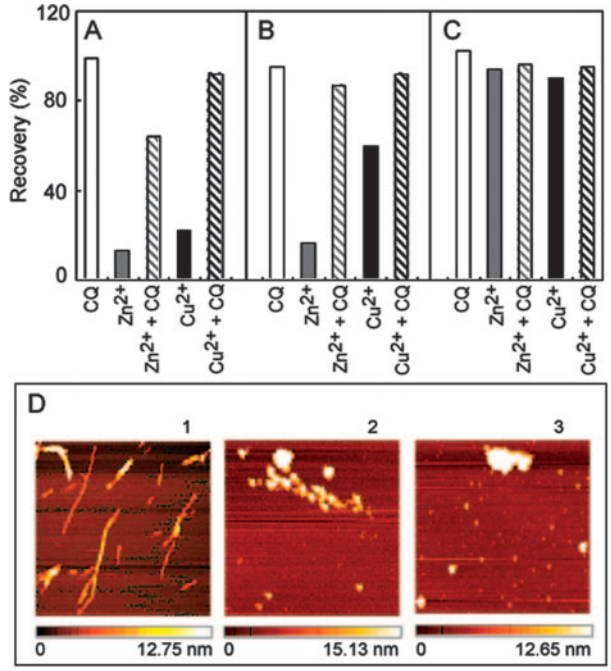

FIG. 1. **Effect of buffer conditions and CQ on Cu^{2+}- or Zn^{2+}-induced aggregation of Aβ$_{1-40}$.** Buffers used were 20 mM HEPES buffer, pH 6.6, containing 100 mM NaCl (*A*), 20 mM HEPES buffer, pH 7.4, containing 100 mM NaCl (*B*), and 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl (buffer A) (*C*). The final concentrations of Cu^{2+}, Zn^{2+}, and CQ were 50, 50, and 100 μM, respectively. *D*, atomic force microscopy images (area corresponds to 2 μm^2) of amyloid fibrils of Aβ$_{1-40}$ (*image 1*) and the aggregates of Aβ$_{1-40}$ formed in 20 mM HEPES buffer, pH 7.4, containing 100 mM NaCl and either 50 μM Zn^{2+} (*image 2*) or 50 μM Cu^{2+} (*image 3*).

Importantly, metal ion-induced aggregation of Aβ$_{1-40}$ depended on buffer conditions. In buffer A, we did not observe metal ion-induced loss of Aβ$_{1-40}$ (Fig. 1*C*). Furthermore, sedimentation velocity measurements showed that the metal ion-treated or untreated Aβ$_{1-40}$ do not exhibit significant change in their sedimentation property (Fig. 2). The apparent sedimentation coefficients of Aβ$_{1-40}$ in buffer A alone and in the presence of 50 μM Zn^{2+} and 50 μM Cu^{2+} were calculated to be 0.96, 0.86, and 1.05 *s*, respectively. Thus, our result shows that the metal ions do not promote significant aggregation of Aβ$_{1-40}$ in buffer A.

One possible reason for the observation made in buffer A could be that the formation of the phosphate complexes of these metal ions prevents their interaction with the Aβ peptide. However, our following results on the effects of Cu^{2+} and Zn^{2+} on the fibril growth and β-sheet formation of the peptide indicate that the peptide interacts with these metal ions in buffer A. Moreover, Cu^{2+} binding to the peptide in buffer A is further supported by the fluorescence quenching studies presented later on in this study. Thus, taking together these evidences, we presume that the observed lack of metal ion-induced aggregation in buffer A is not due to the lack of metal ion binding. Rather, it represents the conditions in which the metal ion-bound peptide exhibits less or no aggregation. In this context, it is important to note that two different laboratories have earlier made contradictory observations on the susceptibility of Aβ peptide toward the Zn^{2+}-induced aggregation (23, 25). Huang *et al.* (26) clarified that the susceptibility of the Aβ peptide to the Zn^{2+}-induced aggregation is sensitive to "complex factors in buffer milieu that impact upon the peptide's

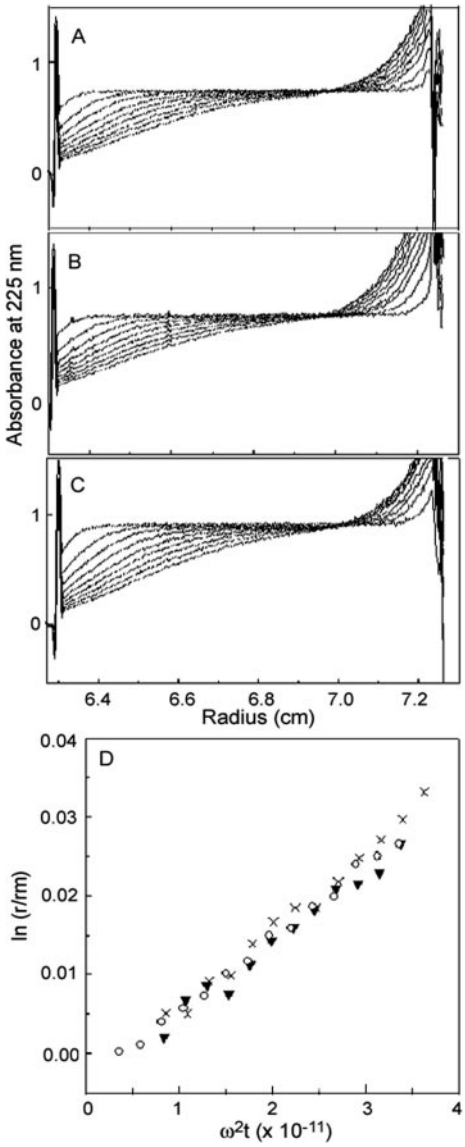

FIG. 2. **Effects of metal ions on sedimentation property of Aβ$_{1-40}$.** *A–C* represent scans of boundary movement of 30 μM Aβ$_{1-40}$ in buffer A alone and in the presence of 50 μM Zn^{2+} and 50 μM Cu^{2+}, respectively, in an analytical ultracentrifugation experiment. *D*, analysis of the sedimentation pattern of Aβ$_{1-40}$. The In(*r*/*rm*) *versus* ω^2t plot for the Aβ$_{1-40}$ in buffer A alone (○) and in the presence of 50 μM Zn^{2+} (▼) and 50 μM Cu^{2+} (×) is shown.

conformation and polymerization state." Therefore, interestingly, our observation that metal ion-induced aggregation is less pronounced in buffer A is useful to understand the relative fibrillogenic propensities of the metal-free (apo) and metal-bound (holo) Aβ$_{1-40}$.

Effect of Cu^{2+}, Zn^{2+}, and Fe^{3+} on the Fibril Growth of Aβ$_{1-40}$—The relative fibrillogenic propensities of the apo- and holo-forms of Aβ peptides have not been understood so far. Since Cu^{2+} or Zn^{2+} does not induce detectable aggregation in buffer A, this system is useful to address the issue. We have investigated the effects of Cu^{2+}, Zn^{2+}, and Fe^{3+} on the amyloid fibril growth of Aβ$_{1-40}$ in buffer A (pH 7.4). Even at substoichiometric concentrations, Cu^{2+} retarded the fibril growth (Fig. 3*A*). At a molar ratio less than 0.4 of the metal ion to Aβ$_{1-40}$, Cu^{2+} increased the time lag of the onset of the fibril growth (despite providing the fibril seed for nucleation). At the molar ratio more than or equal to 0.4, fibril growth was not observed significantly during the observed time period. However, the fibril growth occurred slowly to the extent of the control (without the metal ion) by 2 days of incubation at the molar ratio less than 0.6 (data not shown). At the molar ratio of 1.0, Cu^{2+} prevented the fibril growth completely; there was no fibril growth even after 5 days (data not shown). Zn^{2+} also exhibited a similar effect in preventing the amyloid fibril growth of Aβ$_{1-40}$ (Fig. 3*B*). On the other hand, Fe^{3+} did not prevent the fibril growth of Aβ$_{1-40}$ at concentrations comparable with those of Cu^{2+} or Zn^{2+} (Fig. 3*B*) as well as at its molar ratio as high as 4.0 (data not shown).

We examined by TIRFM the samples of ThT-bound fibril seed, the Aβ amyloid fibrils, and the samples of the Aβ peptide and the seed incubated along with Cu^{2+} or Fe^{3+} (Fig. 3*C*). Fibril growth of Aβ$_{1-40}$ occurred to the order of a few micrometers in buffer A alone (Fig. 3*C*, *image 2*) and even in the presence of 0.2 mM Fe^{3+} (Fig. 3*C*, *image 3*). On the other hand, we could see only fibril seeds in Cu^{2+}-treated (Fig. 3*C*, *image 4*) or Zn^{2+}-treated (data not shown) samples. Thus, Cu^{2+} or Zn^{2+} but not Fe^{3+} prevents the fibril growth of Aβ$_{1-40}$.

Cu^{2+} and Zn^{2+} Prevent the β-Sheet Formation—Amyloid fibrils exhibit characteristic well ordered cross-β-sheet structure (40, 41). Aβ$_{1-40}$ freshly dissolved in buffer A exhibited random coil conformation as revealed in its far UV-CD spectrum (Fig. 4, *curve 1*). Incubation of the peptide in the presence of fibril seeds for 80 min generated a characteristic far-UV CD spectrum for β-sheet structure with a minimum around 218 nm (Fig. 4, *curve 2*). Such an association-induced generation of β-sheet structure also occurred in the presence of Fe^{3+} (Fig. 4, *curve 3*). However, induction of β-sheet structure did not occur in the presence of Cu^{2+} or Zn^{2+} (Fig. 4, *curves 4* and *5*). Thus, Cu^{2+} or Zn^{2+} but not Fe^{3+} prevents the generation of β-sheet structure, consistent with the effects on fibril growth monitored by ThT fluorescence (Fig. 3).

Copper Binding to Aβ$_{1-40}$ in Buffer A—Prevention of the fibril growth and the association-induced β-sheet formation shows that the metal ions, Cu^{2+} and Zn^{2+}, interact with the Aβ peptide in buffer A, where phosphate complexation of the metal ions is also favored. We further investigated the Cu^{2+} binding by fluorescence spectroscopy.

The sequence of Aβ$_{1-40}$ does not contain any tryptophan residue but a single tyrosine residue at position 10 in addition to two phenylalanine residues at positions 19 and 20. The intrinsic fluorescence of the peptide thus predominantly represents the fluorescence of the sole tyrosine residue upon exciting at 280 nm. The intrinsic fluorescence of Aβ$_{1-28}$ is known to be quenched upon binding to Cu^{2+} (20). We have investigated the effect of the metal ions on the intrinsic fluorescence of Aβ$_{1-40}$ peptide. The fluorescence intensity of the peptide progressively decreased upon the addition of the paramagnetic ion, Cu^{2+} (Fig. 5*A*). This observed quenching of the fluorescence is not due to the general collisional quenching of the fluorescence by the paramagnetic ion, Cu^{2+}, as the fluorescence of *N*-acetyl tyrosine amide was not significantly quenched under the concentration range of the metal ion used (Fig. 5*B*). Thus, the specific quenching of the intrinsic fluorescence of Aβ$_{1-40}$ observed in buffer A is due to the specific binding of Cu^{2+}, leading to proximal interactions between the quencher (Cu^{2+}) and the fluorophore (the tyrosine residue of the peptide). Using the fluorescence quenching data (Fig. 5*C*), the dissociation constant (K_d) of copper binding to Aβ$_{1-40}$ was calculated to be $\sim 8 \times 10^6$ M. The addition of the diamagnetic ion, Zn^{2+}, however, did not lead to quenching of the fluorescence of Aβ$_{1-40}$ (Fig. 5*C*). Thus, the fluorescence experiment shows that Cu^{2+} binds to the Aβ peptide in buffer A, under the condition in which the metal ion-induced prevention of fibril growth but not the metal ion-induced aggregation was observed. We presume that Zn^{2+} also interacts with or binds to Aβ$_{1-40}$ in buffer A

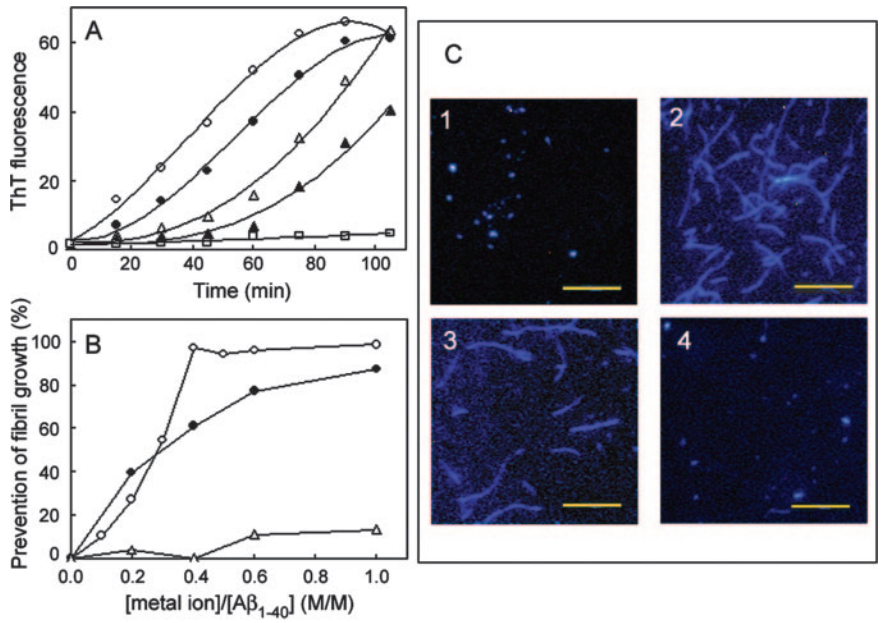

FIG. 3. **Effect of metal ions on the amyloid fibril growth of Aβ$_{1-40}$.** *A*, the amyloid fibril growth of Aβ$_{1-40}$ (50 μM) in buffer A in the absence (○) and in the presence of 5 (●), 10 (△), 15 (▲), and 20 μM (□) Cu^{2+}. *B*, the percentage of prevention of the fibril growth as a function of the molar ratio of metal ion to Aβ$_{1-40}$. Metal ions used are Cu^{2+} (○), Zn^{2+} (●), and Fe^{3+} (△). The percentage of prevention of the fibril growth was calculated using the formula, $[1 - (F_{m90} - F_{m0})/(F_{90} - F_0)] \times 100$, where F_0 and F_{m0} are the ThT fluorescence intensities at zero time in the absence and in the presence of the metal ions and F_{90} and F_{m90} are the fluorescence intensities after a 90-min incubation in the absence and in the presence of the metal ions, respectively. *C*, TIRFM images of fibril seeds (*image 1*); fibrils formed in buffer A alone (*image 2*); and the Aβ$_{1-40}$ samples incubated along with fibril seeds and 0.2 mM Fe^{3+} (*image 3*) and 30 μM Cu^{2+} (*image 4*). The *yellow bar* in the images represents 5 μm.

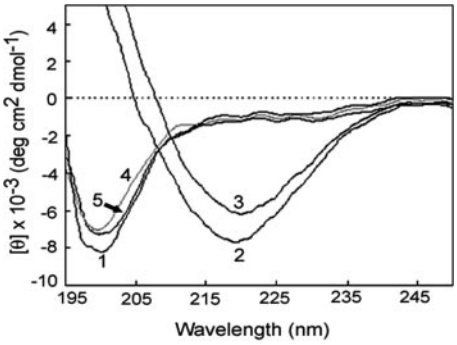

FIG. 4. **Effect of metal ions on the fibril growth-associated generation of β-sheet.** Far-UV CD spectra of Aβ$_{1-40}$ peptide (*curve 1*) and the sample of the Aβ peptide (50 μM) incubated at 37 °C with 5 μg/ml fibril seed for 80 min in the absence (*curve 2*) and presence of 200 μM Fe^{3+} (*curve 3*), 50 μM Zn^{2+} (*curve 4*), or 30 μM Cu^{2+} (*curve 5*) are shown.

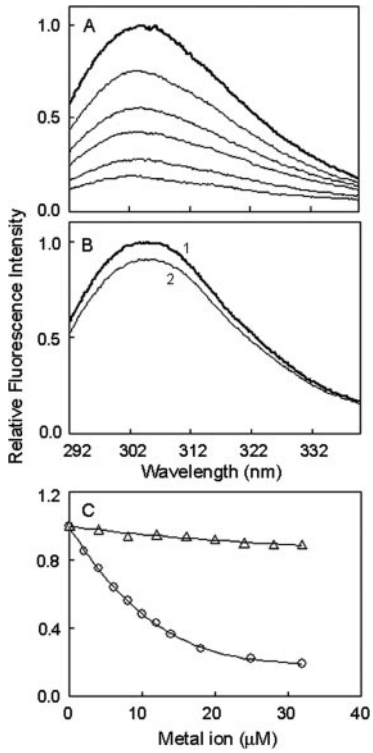

FIG. 5. **Cu^{2+} binding to Aβ$_{1-40}$ in Buffer A monitored by quenching of the intrinsic fluorescence of the peptide.** *A*, intrinsic fluorescence spectra of 10 μM Aβ$_{1-40}$ (*from top to bottom*) in the absence and in the presence of 4, 8, 12, 18, 32 μM Cu^{2+}, respectively. *B*, the fluorescence spectra of *N*-acetyl tyrosine amide in the absence (*curve 1*) and in the presence (*curve 2*) of 50 μM Cu^{2+}. *C*, the relative change in the fluorescence intensity at 304 nm of Aβ$_{1-40}$ as a function of increasing concentrations of Cu^{2+} (○) and Zn^{2+} (△). The samples were excited at 280 nm.

since Zn^{2+} behaved similarly to Cu^{2+} on the fibril growth of Aβ$_{1-40}$ (Fig. 3*B*)

Switching On/Off the Fibril Growth—One of the reasons for our observation that fibril growth is prevented by the metal ions is that the metal ions destabilize the fibrils. However, incubation of the fibrils of the Aβ peptide with the metal ions at 37 °C did not result in significant dissociation of the fibrils as judged by ThT binding (data not shown), thus ruling out the possibility of fibril instability as one of the reasons for the observed metal-induced prevention of the fibril growth. Since Cu^{2+}-treated fibrils are stable but Cu^{2+} prevents the fibril growth, it was possible to switch on/off the fibril growth by switching Aβ$_{1-40}$ to its fibrillogenic apo- and non-fibrillogenic holo-forms (Fig. 6). The results show that one of the key factors determining the Aβ fibril growth is the environmental concentrations of metal ions.

Metal Ion-dependent Effects of CQ on the Fibril Growth—The antibiotic CQ, which can bind both Cu^{2+} and Zn^{2+} with the same stoichiometry but with different coordination geometry (33), has been proposed to serve as a potential drug for AD (8, 31, 32). It would be pertinent to study the effects of CQ on the Aβ amyloid fibril growth as well as on the metal ion-induced suppression of the fibril growth.

CQ alone did not affect the fibril growth of Aβ$_{1-40}$ significantly (Fig. 7*A*). The addition of CQ or EDTA to the sample of Cu^{2+}-treated Aβ$_{1-40}$ led to resumption of the Cu^{2+}-suppresed fibril growth (Fig. 7*B*). Interestingly, the addition of EDTA, but not CQ, led to resumption of the fibril growth suppressed by Zn^{2+} (Fig. 7*C*). Since 8-hydroxyquinoline derivatives are well known transition metal ion chelators (33, 34) and CQ forms complexes with both Zn^{2+} and Cu^{2+} with a similar stoichiometry of 2:1 (33), it is unlikely that the lack of resumption of the Zn^{2+}-suppressed fibril growth is because CQ does not chelate

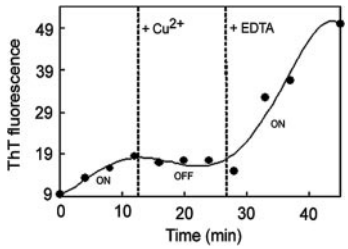

FIG. 6. **Switching on/off the fibril growth of Aβ$_{1-40}$ by Cu^{2+} and EDTA monitored by ThT fluorescence.** The *left vertical dashed line* represents the addition of 30 μM Cu^{2+}, and the *right vertical line* represents the addition of 50 μM EDTA. Buffer A was used.

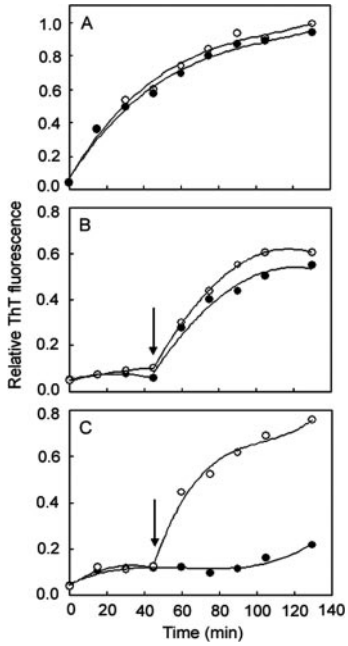

FIG. 7. **Effects of metal chelators on the metal ion-induced suppression of the fibril growth of Aβ$_{1-40}$.** *A*, the effect of CQ on the amyloid fibril growth of Aβ$_{1-40}$. The fibril growth in the absence (○) and presence (●) of 160 μM CQ. *B*, the effect of the metal chelators, 100 μM EDTA (○) and 160 μM CQ (●), on the 50 μM Cu^{2+}-suppressed fibril growth. *C*, the effect of 100 μM EDTA (○) and 160 μM CQ (●) on the 50 μM Zn^{2+}-suppressed fibril growth. The *arrows* indicate the addition of chelators. The final concentration of methanol due to the addition of CQ in this experiment was ~4% (this did not affect the fibril growth significantly).

Zn^{2+}. Since complexes of 8-hydroxyquinoline derivatives with Zn^{2+} are relatively less stable when compared with those with Cu^{2+} (33, 34), a second possibility could be that the possible lesser stability of the Zn^{2+}-CQ complex may lead to shuttling of Zn^{2+} between CQ and the Aβ peptide, which may transiently increase the population of holo-form of Aβ$_{1-40}$ that is less competent for fibril growth. The third possibility is that the Zn^{2+}-CQ complex exhibits novel retardation effect on fibril growth. We, therefore, attempted to verify these possibilities as follows.

At 10 μM Zn^{2+}, the fibril growth of the peptide was prevented only partially (Fig. 8*A*). Since CQ itself does not exhibit significant effect on the fibril growth, the addition of CQ to the sample should not have any effect in the presence of Zn^{2+} if the first possibility of the lack of metal chelation by CQ is valid. If the second possibility is the primary reason, then in the presence of Zn^{2+} and CQ, the fibril growth profile would be expected to be in between that of the control and that of the sample in the presence of Zn^{2+} alone. Surprisingly, the addition of CQ led to further retardation of the fibril growth, indicating a synergistic effect of CQ and Zn^{2+} on the fibril growth

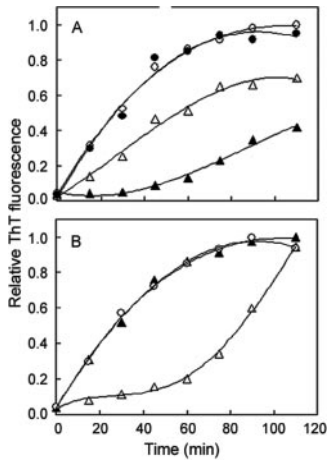

FIG. 8. **Distinct effects of CQ complexation of Zn^{2+} and Cu^{2+} on the fibril growth of Aβ$_{1-40}$.** *A*, synergistic effect of clioquinol and Zn^{2+} in retarding the fibril growth of Aβ$_{1-40}$. *B*, effect of CQ and Cu^{2+} on the Aβ$_{1-40}$ fibril growth. The conditions of amyloid fibril growth of Aβ$_{1-40}$ were in buffer A alone (○), in the presence of 20 μM CQ(●), 10 μM metal ions (△), or a mixture of 20 μM CQ and 10 μM respective metal ion (▲).

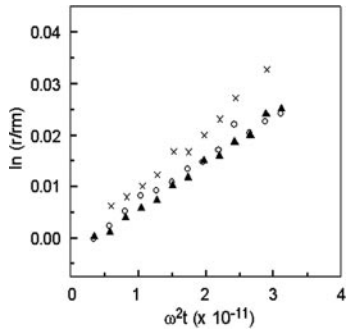

FIG. 9. **Analysis of the sedimentation pattern of Aβ$_{1-40}$ in the presence of CQ complexes of metal ions.** The ln(*r*/*rm*) *versus* ω^2t plot for the Aβ$_{1-40}$ in buffer A alone (○) and in the presence of 50 μM Zn^{2+} and 100 μM CQ (▲) and 50 μM Cu^{2+} and 100 μM CQ (✕). The apparent sedimentation coefficients of the peptide in buffer alone, in the presence of Zn^{2+}-CQ and Cu^{2+}-CQ, were calculated to be 0.9, 0.88, and 1.2 *s*, respectively.

of Aβ$_{1-40}$ (Fig. 8*A*). On the other hand, we did not observe such synergistic effect of CQ in the presence of Cu^{2+}; CQ completely eliminates the suppressive effect of Cu^{2+} on fibril growth (Fig. 8*B*).

From the observed synergistic effect of CQ and Zn^{2+}, it appears that the third possibility is responsible for the observed phenomenon of distinct effects of CQ on the Cu^{2+}- and Zn^{2+}-suppressed fibril growth; Zn^{2+}-CQ complex, but not Cu^{2+}-CQ complex, retards the fibril growth. Sedimentation velocity experiments of the Aβ peptide in the absence and presence of metal ions and CQ revealed no significant changes (Fig. 9), suggesting that metal ion-CQ complexes do not promote other non-fibrillar aggregates not detected by ThT fluorescence. Thus, our results demonstrate that the effect of CQ on the fibril growth process is metal ion-dependent.

DISCUSSION

There has been an increasing interest in the role of transition metal ions in AD (8–10) as Aβ peptides exhibit high affinity binding to Cu^{2+} and Zn^{2+} (16–22). Our study provides two important findings toward the understanding of the role of metal ions and metal ion chelators in AD.

Firstly, our study demonstrates that the soluble holo-forms (Cu^{2+}- or Zn^{2+}-bound) of Aβ$_{1-40}$ are less fibrillogenic or non-fibrillogenic in nature, which was not understood earlier. Al-

though Fe^{3+} is also found at high concentration in AD brain (12), it does not exhibit a significant effect on the fibrillogenic propensity of Aβ$_{1-40}$. It appears that high affinity binding of Cu^{2+} and Zn^{2+} may actually be beneficial as it does not favor fibril formation. On the other hand, high concentrations of metal ion under certain buffer conditions, as shown in Fig. 1, lead to aggregation and precipitation of the peptide, which may be toxic (29, 30).

The second important finding is that CQ exhibits unique metal ion-dependent effects on the fibril growth of the Aβ$_{1-40}$. Chelation of the metal ions such as Cu^{2+} and Zn^{2+} by EDTA leads to conditions favorable for amyloid fibril growth. Animal experiments with CQ have provided encouraging results as a potential drug (8, 31, 32, 36). Our *in vitro* study shows that the addition of CQ leads to resumption of Cu^{2+}-suppressed but not Zn^{2+}-suppressed amyloid fibril growth of Aβ$_{1-40}$. CQ and Zn^{2+} together exhibit synergistic retardation of the fibril growth, suggesting that retardation of fibril growth by the Zn^{2+}-CQ complex is responsible for the observed distinct effects of CQ on the Cu^{2+}- and Zn^{2+}-suppressed fibril growth. However, we cannot completely rule out the possibility of shuttling of Zn^{2+} between CQ and Aβ$_{1-40}$ due to the possible lesser stability of Zn^{2+} complexes of 8-hydroxyquinoline derivatives (33, 34), leading to the transient formation of the less fibrillogenic holo-form. Both of these mechanisms should prove advantageous for the therapeutic use of CQ in AD. Monomeric Aβ is not toxic to the cells (29, 30, 35). Toxicity has been reported to be associated with β-sheet generation and fibril formation (35). On the other hand, granular aggregates, formed before fibril formation, were found be more toxic than the fibrils, although the toxicity depends on the size of the aggregates (29, 30). In this respect, CQ can disaggregate the metal-induced aggregates of Aβ$_{1-40}$ under certain buffer conditions (Fig. 1) and, along with Zn^{2+}, it can retard fibril growth (Figs. 5 and 6).

Although our results suggest that the Zn^{2+}-complex, but not the Cu^{2+}-complex of CQ, retards the fibril growth of Aβ$_{1-40}$, how the complexes distinctly affect fibril propagation is not clear. We consider that the difference in the observed coordination geometry of the complexes of Cu^{2+} and Zn^{2+} (33) may have some role to play. The planar nature of the complex of Cu^{2+} may lead to stacking of the aromatic ring, promoting self-association of the complex. Such stacking is not favorable due to the fifth coordination involving a water molecule as well as due to puckered configuration of the Zn^{2+} complex of CQ (33), which can increase the availability of the complex molecule to interact or interfere in the amyloid fibril growth of the Aβ peptide, probably through hydrophobic interactions.

We conclude that Cu^{2+} and Zn^{2+}, but not Fe^{3+}, significantly prevent the amyloid fibril growth of Aβ$_{1-40}$. Our study demonstrates for the first time that the soluble holo-form of the peptide is non-fibrillogenic in nature and that the fibril growth of the peptide can be switched on/off by switching the molecule between its apo- and holo-forms. CQ exhibits distinct effects on the Cu^{2+}- and Zn^{2+}-induced suppression of the fibril growth. Particularly, complexation of Zn^{2+} with CQ may have dual beneficial effects in not only disaggregating the metal ion-induced aggregates but also in retarding the fibril growth of Aβ$_{1-40}$. Our study should prove useful in understanding the role of the transition metal ions in amyloid fibril propagation of Aβ peptides in AD and provide information concerning the use of metal chelators, particularly CQ, as potential drugs for AD.

Acknowledgments—B. Raman thanks Dr. T. Ramakrishna and Dr. Ch. Mohan Rao, Centre for Cellular and Molecular Biology, India for useful discussions.

REFERENCES

1. Hardy, J., and Selkoe, D. J. (2002) *Science* **297,** 353–356
2. Selkoe, D. J. (1997) *Science* **275,** 630–631
3. Glenner, G. G., and Wong, C. W. (1984) *Biochem. Biophys. Res. Commun.* **120,** 885–890
4. Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., Mc Donald, B. L., and Beyreuther, K. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4245–4249
5. Shoji, M., Golde, T., Ghiso, J., Cheung, T., Estus, S., Shaffer, L., Cai, X., McKay, D., Tintner, R., Frangione, B., and Younkin, S. (1992) *Science* **258,** 126–129
6. Busciglio, J., Gabuzda, D., Matsudaira, P., and Yankner, B. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2090–2096
7. Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D., Lieberberg, I., and Schenk, D. (1992) *Nature* **359,** 325–327
8. Bush, A. I. (2003) *Trends Neurosci.* **26,** 207–214
9. Gnjec, A., Fonte, J. A., Atwood, C., and Martins, R. N. (2002) *Front Biosci.* **7,** d1016–d1023
10. Doraiswamy, P. M., and Finefrock, A. E. (2004) *Lancet Neurol.* **3,** 431–434
11. Sparks, D. L., and Schreurs, B. G. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 11065–11069
12. Lovell, M. A., Robertson, J. D., Teesdale, W. J., Campbell, J. L., and Markesbery, W. R. (1998) *J. Neurol. Sci.* **158,** 47–52
13. Bayer, T. A., Schafer, S., Simons, A., Kemmling, A., Kamer, T., Tepest, R., Eckert, A., Schussel, K., Eikenberg, O., Sturchler-Pierrat, C., Abramowski, D., Staufenbiel, M., and Multhaup, G. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 14187–14192
14. Maynard, C. J., Cappai, R., Volitakis, I., Cherny, R. A., White, A. R., Beyreuther, K., Masters, C. L., Bush, A. I., and Li, Q. X. (2002) *J. Biol. Chem.* **277,** 44670–44676
15. White, A. R., Reyes, R., Mercer, J. F., Camakaris, J., Zheng, H., Bush, A. I., Multhaup, G., Beyreuther, K., Masters, C. L., and Cappai, R. (1999) *Brain Res.* **842,** 439–444
16. Atwood, C. S., Scarpa, R. C., Huang, X., Moir, R. D., Jones, W. D., Fairlie, D. P., Tanzi, R. E., and Bush, A. I. (2000) *J. Neurochem.* **75,** 1219–1233
17. Dong, J., Atwood, C. S., Anderson, V. E., Siedlak, S. L., Smith, M. A., Perry, G., and Carey, P. R. (2003) *Biochemistry* **42,** 2768–2773
18. Curtain, C. C., Ali, F., Volitakis, I., Cherney, R. A., Norton, R. S., Beyreuther, K., Barrow, C. J., Masters, C. L., Bush, A. I., and Barnham, K. J. (2001) *J. Biol. Chem.* **276,** 20466–20473
19. Bush, A. I., Pettingell, W. H., Paradis, M.-d., Jr., and Tanzi, R. E. (1994) *J. Biol. Chem.* **269,** 12152–12158
20. Syme, C. D., Nadal, R. C., Rigby, S. E. J., and Viles, J. H. (2004) *J. Biol. Chem.* **279,** 18169–18177
21. Liu, S. T., Howlett, G., and Barrow, C. J. (1999) *Biochemistry* **38,** 9373–9378
22. Miura, T., Suzuki, K., Kohata, N., and Takeuchi, H. (2000) *Biochemistry* **39,** 7024–7031
23. Bush, A. I., Pettingell, W. H., Multhaup, G., Paradis, M., Vonsattel, J. P., Gusella, J. F., Beyreuther, K., Masters, C. L., and Tanzi, R. E. (1994) *Science* **265,** 1464–1467
24. Atwood, C. S. Moir, R. D., Huang, X., Scarpa, R. C., Bacarra, N. M., Romano, D. M., Hartshorn, M. A., Tanzi, R. E., and Bush, A. I. (1998) *J. Biol. Chem.* **273,** 12817–12826
25. Esler, W. P., Stimson, E. R., Jennings, J. M., Ghilardi, J. R., Mantyh, P. W., and Maggio, J. E. (1996) *J. Neurochem.* **66,** 723–732
26. Huang, X., Atwood, C. S., Moir, R. D., Hartshorn, M. A., Vonsattel, J. P., Tanzi, R. E., and Bush, A. I. (1997) *J. Biol. Chem.* **272,** 26464–26470
27. Yoshiike, Y., Tanemura, K., Murayama, O., Akagi, T., Murayama, M., Sato, S., Sun, X., Tanaka, N., and Takashima, A. (2001) *J. Biol. Chem.* **276,** 32293–32299
28. Klug, G. M., Losic, D., Subasinghe, S. S., Aguilar, M. I., Martin, L. L., and Small, D. H. (2003) *Eur. J. Biochem.* **270,** 4282–4293
29. Kayed, R., Head, E., Thompson, J. L., McIntire, T. M., Milton, S. C., Cotman, C. W., and Glabe, C. G. (2003) *Science* **300,** 486–489
30. Hoshi, M., Sato, M., Matsumoto, S., Noguchi, A., Yasutake, K., Yoshida, N., and Sato, K. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 6370–6375
31. Cuajungco, M. P., Faget, K. Y., Huang, X., Tanzi, R. E., and Bush, A. I. (2000) *Ann. N. Y. Acad. Sci.* **920,** 292–304
32. Ritchie, C. W., Bush, A. I., Mackinnon, A., Macfarlane, S., Mastwyk, M., MacGregor, L., Kiers, L., Cherny, R., Li, Q. X., Tammer, A., Carrington, D., Mavros, C., Volitakis, I., Xilinas, M., Ames, D., Davis, S., Beyreuther, K., Tanzi, R. E., and Masters, C. L. (2003) *Arch. Neurol.* **60,** 1685–1691
33. Di Vaira, M., Bazzicalupi, C., Orioli, P., Messori, L., Bruni, B., and Zatta, P. (2004) *Inorg. Chem.* **43,** 3795–3797
34. Lane, T. J., Sam, C. S. C. A., and Kandathil, A. J. (1960) *J. Am. Chem. Soc.* **82,** 4462–4465
35. Lorenzo, A., and Yankner, B. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12243–12247
36. Cherny, R. A., Atwood, C. S., Xilinas, M. E., Gray, D. N., Jones, W. D., McLean, C. A., Barnham, K. J., Volitakis, I., Fraser, F. W., Kim, Y., Huang, X., Goldstein, L. E., Moir, R. D., Lim, J. T., Beyreuther, K., Zheng, H., Tanzi, R. E., Masters, C. L., and Bush, A. I. (2001) *Neuron* **30,** 665–676
37. Naiki, H., Higuchi, K., Hosokawa, M., and Takeda, T. (1989) *Anal. Biochem.* **177,** 244–249
38. Ban, T., Hamada, D., Hasegawa, K., Naiki, H., and Goto, Y. (2003) *J. Biol. Chem.* **278,** 16462–16465
39. Ban, T., Hoshino, M., Takahashi, S., Hamada, D., Hasegawa, K., Naiki, H., and Goto, Y. (2004) *J. Mol. Biol.* **344,** 757–767
40. Wetzel, R. (2002) *Structure* **10,** 1031–1036
41. Sunde, M., and Blake, C. C. (1998) *Q. Rev. Biophys.* **31,** 1–39

Metal Ion-dependent Effects of Clioquinol on the Fibril Growth of an Amyloid β Peptide

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J. Biol. Chem. 2005, 280:16157-16162.
doi: 10.1074/jbc.M500309200 originally published online February 16, 2005
